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New exploration, DNA sequence data and 3D X-ray microscopy reveal prevalence of live populations and wide variability in shells of *Iberozospeum bellesi* in caves of the Pyrénées-Atlantiques, France (Gastropoda, Ellobioidea, Ellobiidae)

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Abstract

Finding live individuals of the minute, subterranean snail genus, *Iberozospeum* Jochum, Kneubühler, Prieto & Neubert, 2022 is a serendipitous, challenging endeavor. Its diversity has largely been described from caves explored in the Spanish, Cantabrian-Pyrenean part of its range, but only rarely from the French side of the western Pyrenees. Recent exploration has thus targeted cave systems in the Département Pyrénées-Atlantiques to reveal iberozospeid diversity on the southwest, French Pyrenean side. In this study, four live populations were discovered in four different French caves and subjected to integrative taxonomic study, incorporating new genetic data and 3D X-ray microscopic imagery. We show that the new individuals are genetically and morphologically most similar to *I. bellesi* (Gittenberger, 1973) and consequently, assign them to this species.

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3D X-ray image data of type material of *I. bellesi* and that of individuals within this study are presented. The new genetic data is presented within the latest phylogenetic reconstruction of *Iberozospeum* while the prevalence of thriving, live populations of *I. bellesi* is confirmed in multiple caves for the first time in France.

Laburpena

Iberozospeum Jochum, Kneubühler, Prieto & Neubert, 2022, lurpeko barraskilo txikiaren generoko banako bizien aurkitzea ustekabeko eta erronka handiko lana da. Orain arte, genero honen dibertsitatea nagusiki deskribatua izan da banaketa-eremuaren Espainiako aldeko kobazuloetan, Kantauri-Piriniar eskualdean; aldiz, oso gutxitan aztertua izan da Pirinioen mendikatearen mendebaldeko muturrean dagoen muga franko-espainiar eskualdean. Azken esplorazioek, beraz, Pirinio Atlantikoetako departamenduko kobazulo-sistemak izan dituzte xede, Pirinioetako hego-mendebaldeko Frantziako aldean iberozospeidoen dibertsitatea agerian jartzeko, bereziki Ipar-Euskal Herriko lurraldean. Ikerketa honetan, lau populazio aktibo aurkitu dira Frantziako lau kobazulo desberdinetan, eta azterketa taxonomiko integratu baten bidez ikertuak izan dira, datu genetiko berriak erabiliz, baita ere X-izpien teknologiaren bidez sortutako hiru dimentsioko mikroskopia-irudiak baliatuz. Emaitzek erakusten dute aurkitutako banako berri horiek, bai genetikoki bai morfologikoki, *Iberozospeum bellesi* (Gittenberger, 1973) espeziearekin direla antzekotasun eta hurbiltasun handiena dutenak, eta, ondorioz, espezie horri lotuak izan dira. Lan honetan aurkezten dira *I. bellesi*-ren X-izpien teknologiaren bidez sortutako hiru dimentsioko morfologia-irudiak, baita ere ikerketa honetako banakoen deskribapena. Datu genetiko berri horiek *Iberozospeum* generoaren azken berreraikuntza filogenetikoari lotuak eta sailkatuak izan dira, eta lehen aldiz baieztatzen da *I. bellesi*-ren populazio bizi, aktibo eta egonkorrak daudela Frantzian, Pirinio Atlantikoetako departamenduan, bereziki Ipar-Euskal Herriko kobazuloetan.

Keywords

Carychiinae, cave-dwelling species, computer tomography, Franco-Spanish border, microgastropods, molecular phylogenetics, morphology, Nouvelle-Aquitaine, troglobiont ecology

Introduction

Troglobiont micro snails of the genus, *Iberozospeum* Jochum, Kneubühler, Prieto & Neubert, 2022 are rarely found alive in caves of their known range encompassing the Cantabrian-Pyrenean region of northern Spain and the Pyrénées-Atlantiques in southwestern France (Fig. 1). The species, *I. bellesi* (Gittenberger, 1973), is considered uniquely Pyrenean and differentiates morphologically from its eight known Basque-Cantabrian congeners including *I. schaufussi* (von Frauenfeld, 1862); *I. biscaiense* (Gómez & Prieto, 1983); *I. vasconicum* (Prieto, De Winter, Weigand, Gómez & Jochum, 2015); *I. zaldivarae* (Prieto, De Winter, Weigand, Gómez & Jochum, 2015); *I. percostulatum* (Alonso, Prieto, Quiñonero-Salgado & Rolán, 2018); *I. gittenbergeri* (Jochum, Prieto & De Winter, 2019); *I. praetermissum* (Jochum, Prieto & De Winter, 2019), *I. costulatum* Prieto & Jochum, 2022 and the considered fossil, *I. goliath* Prieto & Gómez, 2025. In contrast to its eastern Alpine-Dinaric sister genus *Zospeum* Bourguignat, 1856 multiple species sympatry is not unusual for the genus (Kneubühler et al. 2022).

Although empty shells of *I. bellesi* (Gittenberger, 1973) have frequently been found in southwestern French caves (Dupré 1991, 1994; Tricoche and Origny 2002;

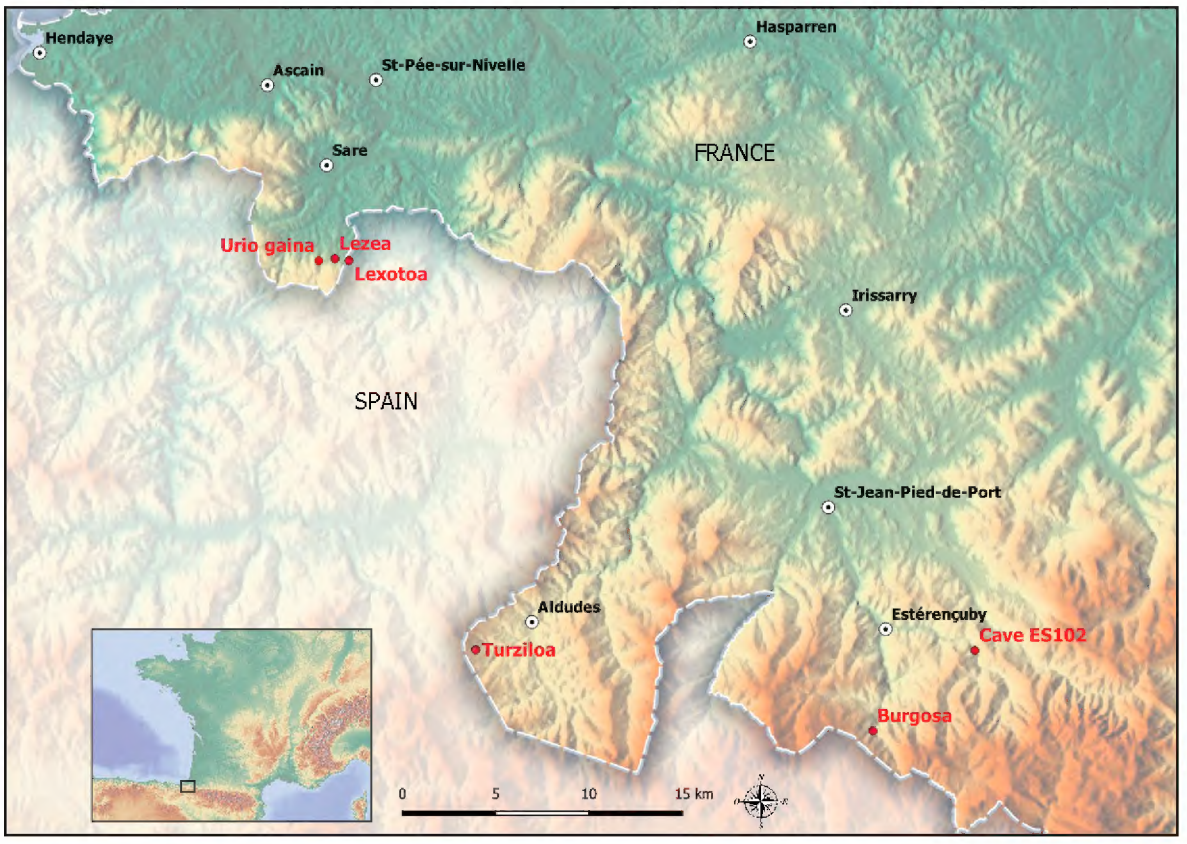

Figure 1. Map showing cave localities of *Iberozospeum bellesi* (Gittenberger, 1973) populations with DNA sequence data in the Basque Country of Spain and France. Cave localities are indicated in red.

Armand et al. 2012; Prieto and Zuazu 2018; Bertrand 2020; Chauvin et al. 2025), only two live populations had been recorded so far (Gargonomy 2018; Kneubühler et al. 2022). Enhanced exploration in April 2025 recently contributed four more to the tally (Chauvin et al. 2025). Of these, only one (Lezea cave) is known for which DNA sequence data exists from the French side of the Franco-Spanish border region (Kneubühler et al. 2022). In the most current phylogenetic study of the genus, only two populations from two proximate non-contiguous cave systems: one from Lexotoa cave (Spain, 100 m eastwards from the Franco-Spanish border) and Lezea cave (in Pyrénées-Atlantiques, less than 1 km from Lexotoa cave) (Fig. 1) were assigned by Kneubühler et al. (2022) to the *I. bellesi* clade. This clade differentiated genetically from the *biscaiense-zaldivarae* clade, but did not however, differentiate genetically from other species using the selected genetic markers (COI, 16S, H3, 5.8 S rRNA +ITS2). Kneubühler et al. (2022) hence suggested that *I. bellesi* likely constitutes a young radiation and that other genetic markers might nonetheless reveal genetic differentiation in *I. bellesi*.

Owing to the scarcity of data from caves on the French side of the Pyrenean chain and the paucity of DNA sequence data for *I. bellesi* in Kneubühler et al. (2022), new biospeleological expeditions (Chauvin et al. 2025) were conducted in March 2024 and April 2025 in karst caves of the Sare-Aïnhoa sector of the Franco-Spanish border region of the Pyrenees and its foothills in the department Pyrénées-Atlantiques. The Sare-Aïnhoa karst region lies about fifteen kilometers from the Atlantic Ocean and spans only a few square kilometers into both France and Spain (Navarra) (Vanara and Besson 2010).

In this work, three new populations sampled in March 2024, are assessed using DNA sequence data in sync with Kneubühler et al. (2022), whereby the sequences from these populations are added to the existing DNA sequence dataset. These new data are integrated into Kneubühler et al. (2022) phylogenetic reconstruction and presented here in context with its iberozospeid congeners (Fig. 2). Hereby, we provide a fundamental integrative presentation of extant members of the *I. bellesi* clade, incorporating image data of type material of *I. bellesi* (RMNH.MOL.234137) in conjunction with that of shells from the new French populations using 3D X-ray microscopy. The new morphological data present an enhanced description of the species, *Iberozospeum bellesi* (Gittenberger, 1973). The geological context, topography and ecology of the caves in the Franco-Spanish border region of the Pyrénées-Atlantiques are described. Live prevalence of the species is confirmed in multiple caves for the first time in France.

Material and methods

Abbreviations of collection depositories

AJC private collection A. Jochum, Germany
MHNG Muséum d'Histoire Naturelle, Geneva, Switzerland
MNHN Muséum National d'Histoire Naturelle, Paris, France
NMBE Natural History Museum, Bern, Switzerland
RMNH Naturalis Biodiversity Center (former RijksMuseum van Natuurlijke Historie), Leiden, The Netherlands
SNSB Zoologische Staatssammlung München, München, Germany

Abbreviations of shell characters

ah aperture height
aw aperture width
hlw height of last whorl
SA spire angle
sh shell height
sw shell width

Individuals investigated in the molecular study

Individuals used for DNA sequence analyses were collected by JC, SG and QW. A total of 7 individuals were imaged, measured and molecularly processed. Sampling locations and the GenBank accession numbers for the obtained mtDNA (COI, 16S) and nDNA (H3, 5.8 S rRNA+ITS2) sequences are given in Table 1. Images and the DNA extracts of the 7 molecularly assessed specimens are housed in the Natural History Museum Bern (NMBE), Bern, Switzerland.

Table 1. Molecularly assessed French cave populations with their inventory numbers, geographic location, individual shell height and GenBank accession numbers for the analyzed mtDNA (COI, 16S) and nDNA (H3, 5.8 S rRNA + ITS2) sequences.

Lab No.	AJC	NMBE No.	Species	Locality	GPS	Shell height (mm)	Genbank COI	Genbank 16S	Genbank H3	Genbank ITS2
3234	2494	MOLL-0585632	*Iberozospeum bellesi*	France, Pyrénées-Atlantiques, Estérençuby, Burgosa cave	43.054852, -1.20068, alt. ca. 530 m	1.41	-	-	-	-
3235	2494	MOLL-0585633	*Iberozospeum bellesi*	France, Pyrénées-Atlantiques, Estérençuby, Burgosa cave	43.054852, -1.20068, alt. ca. 530 m	1.37	-	-	-	-
3236	2493	MOLL-0585635	*Iberozospeum bellesi*	France, Pyrénées-Atlantiques, Aldudes, Turziloa cave	43.083873, -1.462237, alt. ca. 640 m	1.53	PX353354	PX353470	PX400169	-
3237	2493	MOLL-0585636	*Iberozospeum bellesi*	France, Pyrénées-Atlantiques, Aldudes, Turziloa cave	43.083873, -1.462237, alt. ca. 640 m	1.52	PX353355	PX353471	PX400170	-
3238	2498	MOLL-0585638	*Iberozospeum bellesi*	France, Pyrénées-Atlantiques, [Vallée d'Estérenguibel], Estérençuby, cave ES102; lateral gallery	43.096567, -1.136395, alt. ca. 356 m	1.31	PX353356	PX353472	PX400171	-
3239	2498	MOLL-0585639	*Iberozospeum bellesi*	France, Pyrénées-Atlantiques, [Vallée d'Estérenguibel], Estérençuby, cave ES102; lateral gallery	43.096567, -1.136395, alt. ca. 356 m	1.46	PX353357	PX353473	PX400172	PX353469
3240	2497	MOLL-0585641	*Iberozospeum bellesi*	France, Pyrénées-Atlantiques, Sare, cave Urio gaina (station 2)	43.26663, -1.58213, alt. ca. 350 m	1.41	PX353358	PX353474	PX400173	-

DNA sequence analysis

Live specimens were preserved initially in 96% ethanol and later transferred to an 85% ethanol solution. Before DNA extraction, every specimen was imaged under sterile conditions from frontal, lateral and apical view with a Leica DFC425 microscope camera using the image-processing program (IMS Client V15Q4, Imagic, Switzerland). For total DNA extraction the Qiagen Blood and Tissue Kit (Qiagen; Hilden, Germany) was used in combination with a QIAcube extraction robot. Each specimen was placed in a mix of 180 µl ATL buffer and 20 µl Proteinase K. It was then incubated for ca. 4 hours at 56 °C in a heater (Labnet, Vortemp 56, witec AG, Littau, Switzerland). For subsequent DNA extraction the QIAcube extraction robot with the Protocol 430 (DNeasy Blood Tissue and Rodent tails Standard) was used.

In this study, two mitochondrial (mtDNA) markers (COI and 16S) and two nuclear (nDNA) markers (H3, 5.8 S rRNA+ITS2) were investigated. PCR mixtures consisted of 12.5 µl GoTaq G2 HotStart Green Master Mix (Promega M7423), 4.5 µl ddH_2O, 2 µl forward and reverse primer each, and 4 µl DNA template. In Table 2, the respective primer pairs for the PCR are listed. The following PCR cycles were used: for COI the admixture was heated 2 min at 94 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 40 °C and 1 min at 72 °C and finally, 5 min at 72 °C; for 16S the admixture was heated 5 min at 95 °C, followed by 45 cycles of 30 s at 95 °C, 30 s at 48 °C and 45 s at 72 °C, and finally, 5 min at 72 °C; for H3 the admixture was heated 3 min at 95 °C, followed 40 cycles of 1 min at 95 °C, 1 min at 42 °C and 1 min at 72 °C, and finally, 10 min at 72 °C, for 5.8 S rRNA+ITS2 the admixture was heated 1 min at 96 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, and finally, 10 min at 72 °C (SensoQuest Tabcyclet and Techne TC-512, witec AG, Littau, Switzerland). The purification and sequencing of the PCR products was performed by LGC (LGC Genomics Berlin, Germany).

Though successfully sequenced, the DNA content of the individuals from Estérençuby, Burgosa cave (43.054852, -1.20068, alt. 530 m) (NMBE-MOLL-0585632–0585633) was somehow too low. Hence, sequences of these specimens could not be included in this study.

Table 2. Primer designs used for PCR reactions.

Marker	Primer	Sequence	Sequence length (bp)	Reference
COI	LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	680	Folmer et al. 1994
	HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'		
16S	16S ar	5'-CGC CTG TTT ATC AAA AAC AT-3'	440	Simon et al. 1994
	16S br	5'- CCG GTC TGA ACT CTG ATC AT -3'		
H3	H3AD	5'-ATGGCTCGTACCAAGCAGACVGC-3'	380	Colgan et al. 1998
	H3BD	5'-ATATCCTTRGGCATRATRGTGAC-3'		
5.8 S rRNA+ITS2	ITS2ModA	5'-GCTTGCGGAGAATTAATGTGAA-3'	900	Bouaziz-Yahiatene et al. 2017
	ITS2ModB	5'-GGTACCTTGTTCGCTATCGGA-3'		

Phylogenetic analyses

The software package GENEIOUS v9.1.8 (Biomatters Ltd) was used for sequence processing and editing. Maximum Likelihood (ML) topology was estimated with the GENEIOUS RAxML plug-in (Stamatakis 2006) using the rapid bootstrapping setting to compute the best scoring ML tree and 1000 bootstrapping replicates.

We additionally implemented sequences previously used in Kneubühler et al. (2022) for the *I. bellesi* clade (dark blue), encompassing individuals from Lezea cave (NMBE-MOLL-557236) and Lexotoa cave (NMBE-MOLL-557234) (Fig. 2).

Imaging acquisition

Light microscopy (LM)

Digital images of the paratype material RMNH.MOL.234137 (Fig. 3) were taken with a Zeiss Discovery V20 stacking microscope at the Naturalis Biodiversity Center Leiden, NL. Each specimen was captured by a series (ca. 20–55) of images in z-direction (re. 3D Cartesian coordinate system) which were subsequently stacked using the software Zen 3.8 (Carl Zeiss Microscopy GmbH, Jena, Germany).

X-ray microscopy (Micro-CT)

Most specimens were scanned with an XRadia MicroXCT-200 imaging system (1600 projection images, 360° rotation for ca. 90 mins, voxel size ca. 2 µm, voltage 40 kV, current 200 µA) (Carl Zeiss Microscopy GmbH, Pleasanton, CA, United States). The paratype shell of *Iberozospeum bellesi* (RMNH.MOL.234137) was scanned separately using the NeoScan N80 imaging system (mostly 1600 projection images, 360° rotation for 120 min, voxel size 1.8 µm, voltage 50 Kv, current 200 µA). The 16 bit image data sets generated by reconstruction of the tomographic data were cropped, histogram adjusted and converted to 8 bit using VGStudio MAX 2.2 software (Volume Graphics, Heidelberg, Germany). Further 3D graphical procedures were conducted with Amira 6.4 software (Thermo Fischer Scientific, Electron Microscopy Solutions, Hillsboro, Oregon, USA). An initial segmentation step involving the watershed function was used to digitally remove impurities from the shells. Subsequently, manual segmentation for discrimination of external and internal shell structures was applied. Final visualization was conducted using the Volume Rendering module.

Measurements

Shell measurements were made on Micro-CT images following Jochum et al. (2024) using dimension tools of the software Corel Draw 2017 (Corel Corporation, Ottawa, Ontario, Canada). Shell whorl number was counted to the nearest quarter whorl according to Kerney and Cameron (1979). Measurements were taken for each specimen

including, shell height (sh), shell width (sw), aperture height (ah), aperture width (aw), height of last whorl (hlw) and spire angle (SA) (Table 3). Taxonomic inferences in this study are based on fully grown specimens.

Morphological analyses

Following the work of Jochum et al. (2024) significant morphological features (i.e. character states) of shells from each cave were compared to those of the paratype material (Fig. 3), RMNH.MOL.234137 in each of the Micro-CT-scanned perspectives, starting with the aperture frontal view and the long axis vertical (Fig. 4). From this position, each shell was then studied with aperture facing right, apical view, dorsal view, aperture facing left and the ventral side (Fig. 5). The respective cave populations are recorded, including depository catalog numbers, presence of teleoconch sculpture, columellar configuration including presence of structural bulging on the columella (there is no definable lamella in *I. bellesi*) and its alignment relative to the penultimate whorl (aperture facing right, dorsal view and aperture facing left), protoconch and whorl configuration (apical view), upper peristome rim alignment to the penultimate whorl (aperture facing left), peristome thickness and the configuration of the last ¼ coiling of the body whorl in relation to the umbilical depression (ventral view) (Table 4, Figs 6–9).

Geological composition, topography and ecology of cave sites

The sampled caves of the Sare-Aïnhoa sector of the Pyrenean chain (Lezea and Urio gaina) constitute several non-contiguous cavities developed in Lower Cretaceous limestone (Albo-Aptian and Cenomanian stages) (Vanara and Besson 2010). These caves are geographically and geologically considered a single "unit" irrespective of the political boundary separating the two countries (Dachary 2009). Burgosa cave (Esterençuby) is located in the mid-mountain, Urkulu-Mendilaz Massif, a tectonically impacted monoclinal mass formed in Cretaceous sedimentary layers (Maastrichtian, Campanian, Santonian, Coniacian) resting on a Paleozoic base (Devonian) (Dupré 1991). The cave, ES102 (Esterenguibel Valley), comprises a small cavity fed by a temporary stream in Carboniferous limestone (Namurian) in Esterenguibel karst (Dupré 1991). The Turziloa cave has an elevation drop of 15 meters and extends 125 meters (also known as Urziloa, Ithurziloa, or Oyanzelhako Urziloa cave). It is a partially very active cave in the Baïgorry-Aldudes Valley. The region consists of crystalline cement sandstone (quartzite) from the Paleozoic as well as Ordovician schists and dolomite limestone (Dupré 1991).

The caves, ES102, Urio gaina, Burgosa and Turziloa (Figs 10–13) comprise cavities of diverse size and structure. Live *Iberozospeum bellesi* and expired colonies of shells, still attached to their associated habitat of sponge-like mud (Jochum et al. 2012), were found in small chambers measuring approximately 20 meters in length (ES102) (Fig. 10) to subterranean networks extending over 100 meters (Urio gaina) (Fig. 11). The topography of these karst cavities is highly variable, ranging from simple (Burgosa, ES102) (Fig. 12) to more complex networks (Urio gaina, Turziloa). The Turziloa cave (Fig. 13) for

example, is distinguished by the presence of an active subterranean river whereby *I. bellesi* were found toward the end of the system, at the junction of active flow and a fossil gallery, in areas exposed to spray from water flows. Over all, the entrances to the caves in this study are generally small, measuring between 1 and 2 meters in width, though they can reach a moderate size of 5 to 7 meters. The cave entrances have been charted at altitudes ranging from 300 to 650 meters. All photographs of the cave sites were taken by JC.

In reference to the geography versus the geological context of the region, we remark that specimen NMBE 557236 from Lezea cave in Kneubühler et al. (2022, Table 1) is listed as having been collected in Navarra [Spain]. Geologically, Lezea cave is part of the same karst system as the Zugarramurdi caves in Spain (Navarra) but belongs rather, to the municipality of Sare, France (Prieto and Zuazu 2018), albeit it is geographically located just a few hundred meters from the Spanish border.

Results

Our ML analysis revealed that the French populations cluster in the iberozospeid lineage *I. bellesi* (Fig. 2). Here, the only animal delivering the complete dataset is integrated in the cladogram shown by Kneubühler et al. (2022, Fig. 3). Remarkably, the other six specimens with an incomplete marker set always clustered in a polytomy including the Spanish *I. bellesi* populations. For this reason, the species identification is considered reliable. Addition of the new data set resulted in a new arrangement of the lineages. In the analysis of Kneubühler et al. (2022, Fig. 3), the subclade "*zaldivarae* + *biscaiense*" represented the highly

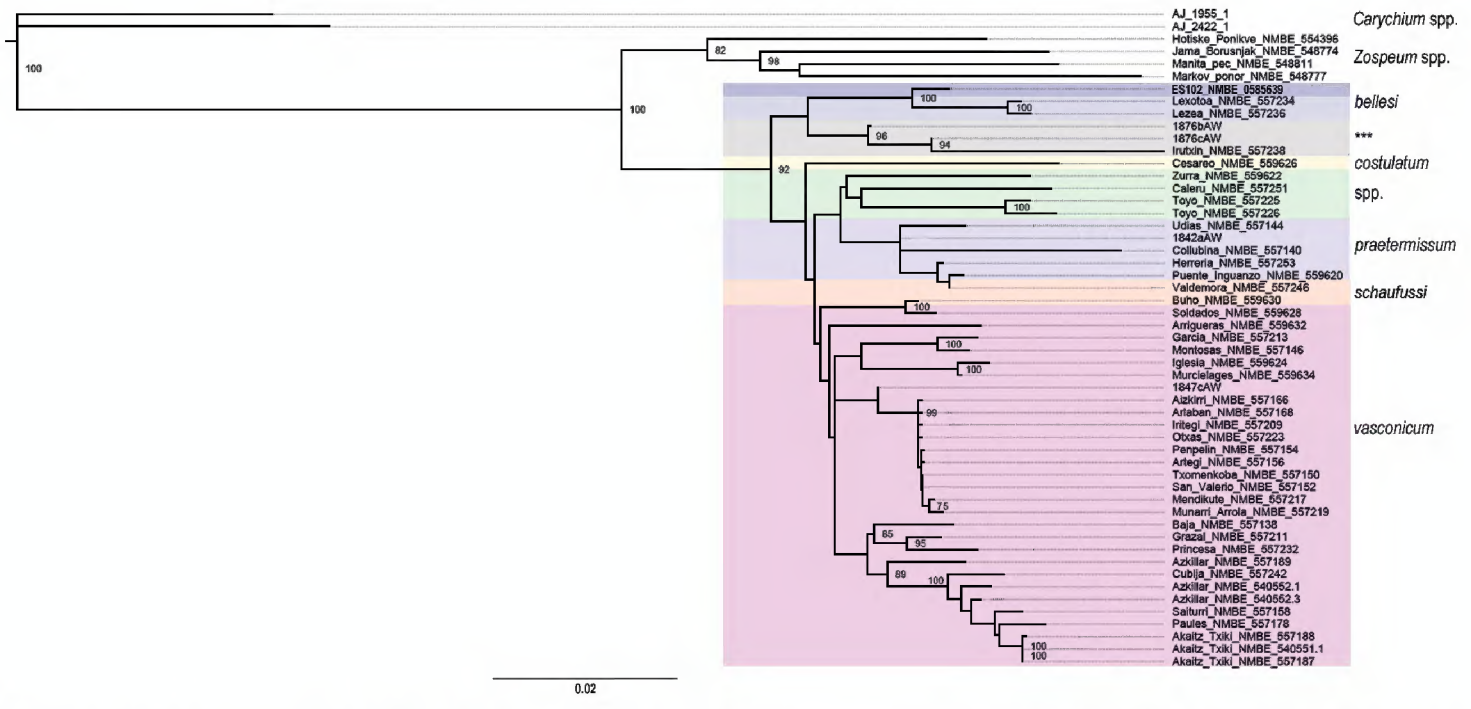

Figure 2. Maximum likelihood (RAxML) tree based on concatenated data set of COI, 16S, H3 and 5.8 S rRNA + ITS2. The outgroup taxa include four Dinaric *Zospeum* specimens and two species of *Carychium*. Only specimens with complete marker sets are included. *** = *I. biscaiense-I. zaldivarae*. The position of the French *I. bellesi* population, NMBE_0585639, from Vallée d'Estérenguibel, Estérençuby cave, ES102 is designated in deep blue. The numbers at the nodes represent the bootstrap support values.

supported sister group to all *Iberozospeum* species investigated. In the current analysis, the *I. bellesi* and the "*I. zaldivarae* + *I. biscaiense*" subclades are united (although only weakly supported) taking over the sister position now (support value > 92). This shows that the current state of knowledge is still rather incomplete and that more precise phylogenetic relationships can be expected when applying other methods and markers.

Measurements of shell characters reveal that these shells are not only minute but demonstrate a narrow range in shell size (sh: 1.17–1.60 mm) and shell width (0.85–1.02 mm) (Table 3). Despite their tininess, *I. bellesi* shells show considerable variation in shell shape and proportions. Since the relationship between shell height (sh) and shell width (sw) defines the slenderness and stoutness of the shell, the ratio of these measurements shows a variation ranging from 1.28 to 1.63, indicating the presence of both relatively squat and markedly elongate shell forms. The shell (NMBE-MOLL-0590059-2) from cave ES102 for example, demonstrates the stoutest shell of this study, which is most comparable to that of the paratype material (RMNH. MOL.234137) from Cueva [del Molino] de Aso, Huesca, Spain (Figs 3–5). In contrast, the individual from Urio gaina (NMBE-MOLL-0590058-1) presents a much slenderer shell form. Aperture dimensions are consistent with aperture height (ah) ranging from 0.45–0.58 mm while aperture width (aw) ranges from 0.45–0.54 mm. The aperture shape ratio (ah/aw) ranges from 0.83 to 1.16 with a ratio ah/aw frequently close to 1, indicating that the aperture scales proportionally with shell size and that the aperture is roughly oval to rounded in shape. The height of the last whorl (hlw) constitutes the major portion of total shell height in all specimens. The hlw/sh ratio ranges from 0.54 to 0.65, indicating that the last whorl consistently makes up more than half the total shell height, reflecting a compact spire and a shell form dominated by the body whorl. Of all the measurements in Table 3, the spire angle (SA) presents the greatest degree of variation, ranging from 51.84° to 70.93°. It clearly defines the key shape in these shells whereby a low SA (ca. 52°) reflects a tall, narrow and pointed spire and a high SA (ca. 71°) defines a short, wide and blunt spire. Of the 9 shells comprising the morphological part of this study, shell NMBE-MOLL-0590057-1 (Burgosa cave) bears the lowest spire angle (very pointed) measuring at 51.84° while that of cave ES102 (NMBE-MOLL-0590059-2) demonstrates the highest at 70.93° (very broad). We remark an apparent negative correlation between shell slenderness (sh/sw) and spire angle (SA), with more slender shells tending to exhibit lower spire angles.

In conjunction with the measurement data, our Micro-CT image data (Table 4, Figs 6–9) reveal minimal variation in morphological characters such as teleoconch sculpture, peristome thickness, columellar configuration, protoconch and whorl configuration, and peristome rim alignment to the penultimate whorl (aperture facing left). On the other hand, there is noticeable differentiation in aperture shape from ovate to roundish-ovate to round with the aperture alignment either oblique to the shell axis or vertically aligned to it. The configuration of the parietal callus is also quite variable in degree of thickness and the extent to which it extends along and onto the body whorl (frontal view). The shells of this study also vary in the configuration of the last ¼ coiling of the body whorl in relationship to the umbilical zone (ventral view). For example, in the paratype shell (RMNH.MOL.234137), the end of the final whorl is very short

Table 3. List of shell measurements denoted by population locality, museum or collection catalog number, and individual specimens assessed per population. Measurements include shell height (sh), shell width (sw), aperture height (ah), aperture width (aw), height of last whorl (hlw) (in mm) and spire angle (SA) (in degrees).

Population locality	Specimen (spm)	sh	sw	ah	aw	hlw	SA
Turziloa cave	NMBE-0590056-1 (AJC-2493-1)	1.42	0.96	0.45	0.54	0.79	54.6
Turziloa cave	NMBE-0590056-2 (AJC-2493-2)	1.47	0.97	0.58	0.51	0.87	60.6
Burgosa cave	NMBE-0590057-1 (AJC-2495-1)	1.58	0.97	0.56	0.53	0.86	51.8
Burgosa cave	NMBE-0590057-2 (AJC-2495-2)	1.38	1.02	0.57	0.49	0.82	61.5
Urio gaina cave (station 1)	NMBE-0590058-1 (AJC-2496-1)	1.6	1.01	0.56	0.54	0.86	52.5
Urio gaina cave (station 1)	NMBE-0590058-2 (AJC-2496-2)	1.45	1.02	0.58	0.54	0.88	64.4
Cave ES102	NMBE-0590059-1 (AJC-2498-1)	1.29	1.01	0.57	0.5	0.77	67.1
Cave ES102	NMBE-0590059-2 (AJC-2498-2)	1.17	0.89	0.49	0.45	0.76	70.9
Cueva de Aso, Huesca	*I. bellesi* RMNH.MOL.234137	1.27	0.85	0.45	0.46	0.77	64

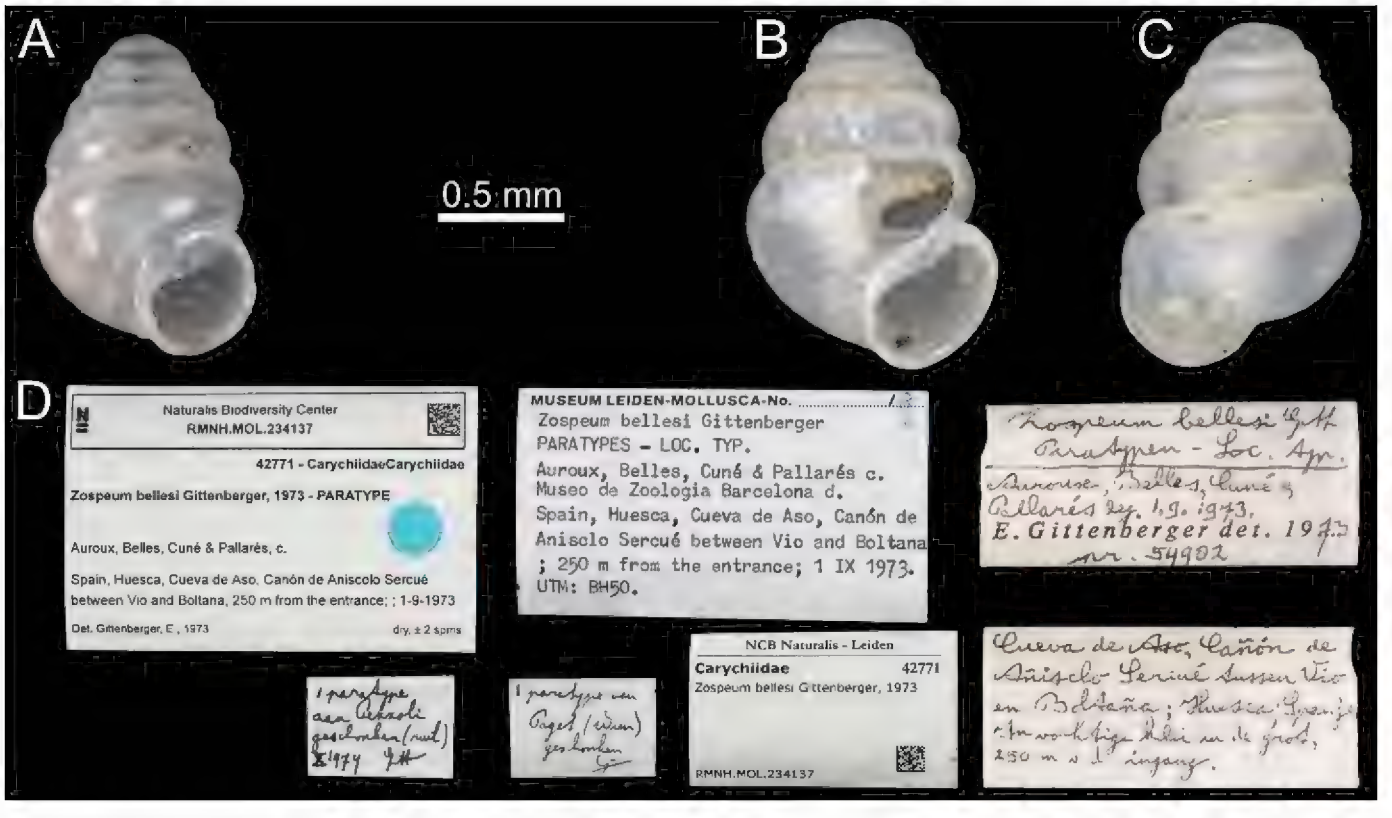
A
B
C
0.5 mm
D
Naturalis Biodiversity Center
RMNH.MOL.234137
42771 - CarychiidaeCarychiidae
Zospeum bellesi Gittenberger, 1973 - PARATYPE
Auroux, Belles, Cuné & Pallarés, c.
Spain, Huesca, Cueva de Aso, Canón de Aniscolo Sercué between Vio and Boltana, 250 m from the entrance; ; 1-9-1973
Det. Gittenberger, E., 1973
dry, ± 2 spms
MUSEUM LEIDEN-MOLLUSCA-No.
Zospeum bellesi Gittenberger
PARATYPES - LOC. TYP.
Auroux, Belles, Cuné & Pallarés c.
Museo de Zoologia Barcelona d.
Spain, Huesca, Cueva de Aso, Canón de Aniscolo Sercué between Vio and Boltana ; 250 m from the entrance; 1 IX 1973.
UTM: BH50.
NCB Naturalis - Leiden
Carychiidae 42771
Zospeum bellesi Gittenberger, 1973
RMNH.MOL.234137

Figure 3. Light microscopic images of *Iberozospeum bellesi* paratype shells (RMNH.MOL.234137) from Cueva [del Molino] de Aso, Huesca, Spain with collection labels. Frontal view of scanned shell via Micro-CT (see also Fig. 5) (**A**), frontal view of a second shell in paratype vial (RMNH.MOL.234137) (**B**), dorsal view of specimen B (**C**), specimen vial labels (**D**). Credit: Frank Loggen, 2025.

and compactly formed, aligning straight next to a narrow umbilical depression (Fig. 5F). Conversely, one shell from Turziloa cave (NMBE-MOLL-0590056-1) shows this part not being compact and straight but rather, extending to the right side of the body

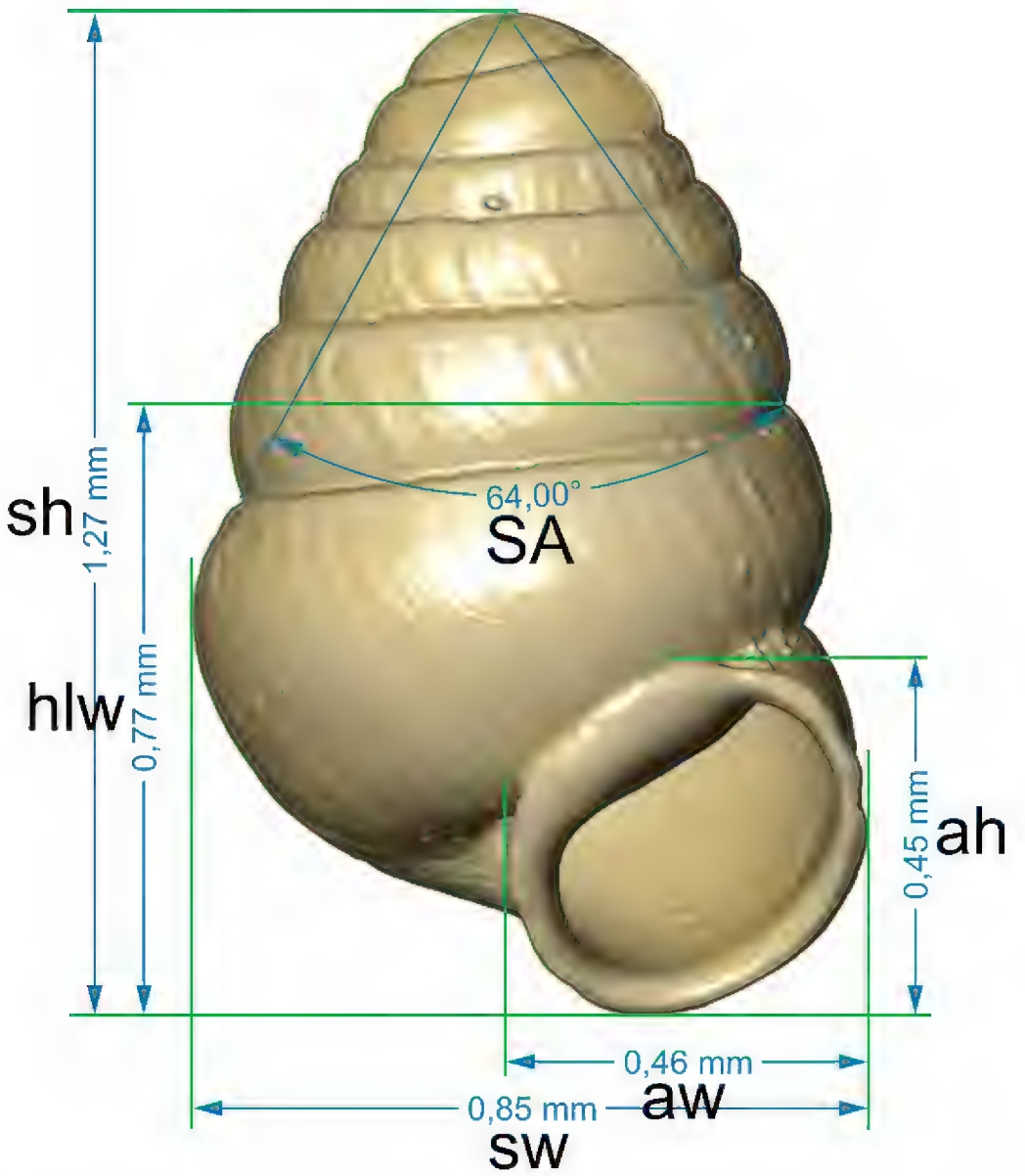

Figure 4. 3D visualization of Micro-CT data of *Iberozospeum bellesi* (Gittenberger, 1973) (RMNH. MOL.234137) with measurements showing opaque shell in frontal view, including shell height (sh), shell width (sw), aperture height (ah), aperture width (aw), height of last whorl (hlw) and spire angle (SA).

whorl, forming a large umbilical depression (ventral view) (Fig. 6F). The second shell from Turziloa cave (NMBE-MOLL-0590056-2) bears rather, a shorter, more compact end part of the final whorl (Fig. 6L), which forms a larger umbilical depression than that present in the paratype shell. A similar contrasting situation is observable with the two shells from Burgosa cave (Fig. 7F, L) and from cave ES102 (Fig. 9F, L) while the ventral sides of the two shells from Urio gaina (NMBE-MOLL-0590058-1–2) (Fig. 8F, L) largely match the same ventral morphological configuration as that of the *I. bellesi* paratype (RMNH.MOL.234137) from Cueva [del Molino] de Aso, Huesca, Spain. We remark that these contrasting differentiating conditions in shells collected in the same cavities of the same caves may well reflect different generations of *I. bellesi* impacted by different environmental influences at different times. Considering that 5 from 9 shells in our morphological study encountered some degree of breakage during the usually non-destructive Micro-CT scanning process, is an indication that these shells are extremely thin and may possibly constitute fossil material. Moreover, Gittenberger (1980) remarked that the shells collected by JGM Raven (in 1978) from Navarra were less fragile than those he described from the type locality in Huesca.

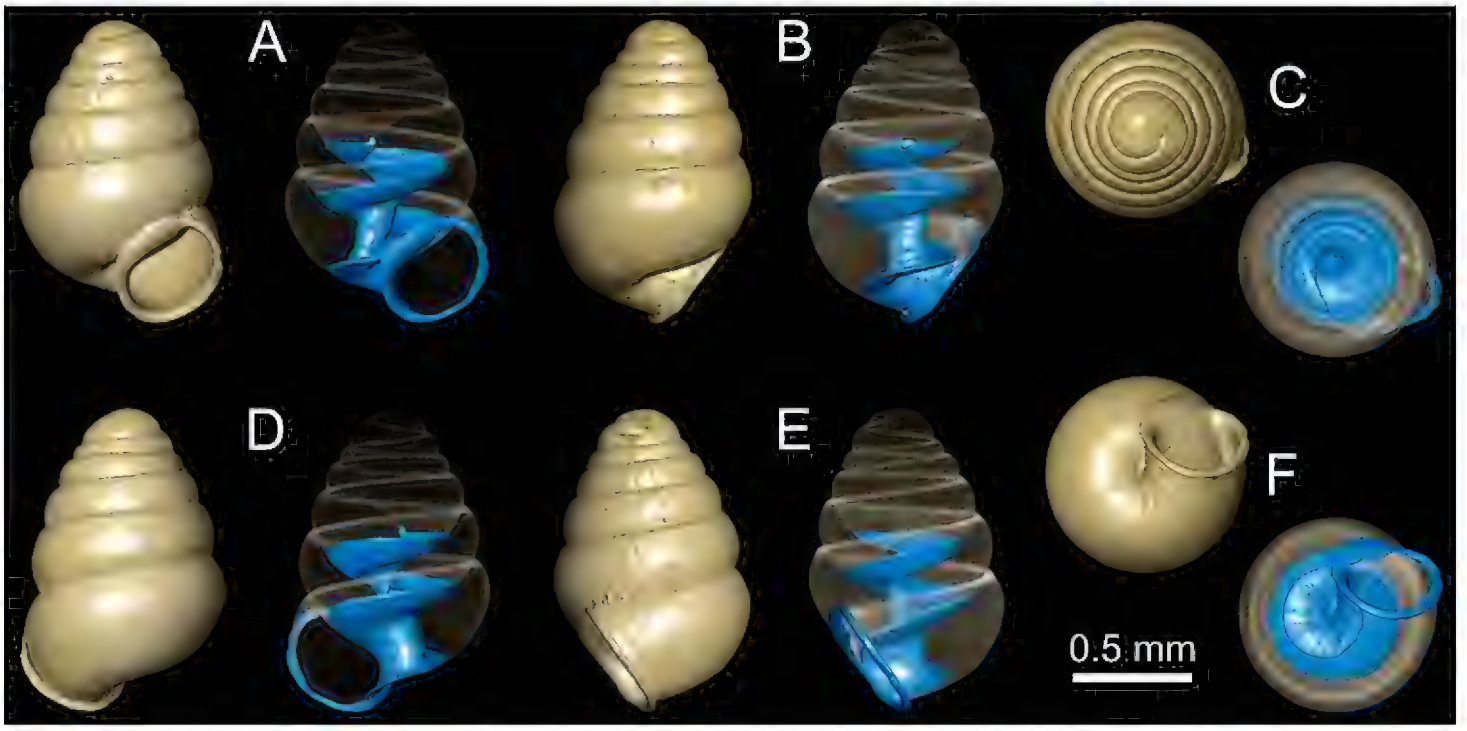

Figure 5. 3D visualizations of Micro-CT data of the paratype *Iberozospeum bellesi* (Gittenberger, 1973) (RMNH.MOL.234137) **A–F.** Shell of Fig. 3A in opaque and semi-translucent reconstructions in frontal view showing aperture alignment (**A**), aperture facing right view showing columellar configuration (**B**), apical view showing protoconch and coiling tightness (**C**), dorsal view (**D**), aperture facing left view showing aperture rim alignment (**E**), umbilical view showing umbilical depression and configuration of last ¼ whorl growth and alignment (**F**).

Hereby, environmental influences may largely factor in the degree of shell thickness of *I. bellesi* in these specific caves of the Franco-Spanish border region.

Ecologically, living *Iberozospeum* were found in areas typically characterized by moist to moderately humid walls, which occasionally exhibit signs of carbonated efflorescence. The runoff from these walls fosters the regular accumulation of organic mud where *I. bellesi* were found in a variety of locations, including cave walls, rock crevices, and the base of walls above the interface to the ground. These sites are characterized by their relative protection from air currents, serving as microhabitats that remain unexposed and prone to continuous, consistent environmental conditions over time. In such habitats, several live individuals were found in areas measuring approximately 3 to 4 square meters (e.g. in ES102). We remark that though ample, empty iberozospeid shell material was collected from other caves in the Pyrénées-Atlantiques during the speleological sampling for this investigation, we assessed only those shells from the live populations within the context of this study.

Discussion

Iberozospeum bellesi is a strictly Pyrenean species still relatively underrepresented in the only molecular investigation of the genus (Kneubühler et al. 2022) but well documented in morphological and distributional assessments of the Basque Country (Dupré 1991; Prieto and Zuazu 2018). In our molecular study, only the population from ES102

Table 4. Major differential diagnostic characters of French *Iberozospeum bellesi* (Gittenberger, 1973) populations revealed by Micro-CT.

Locality & Specimen	Teleoconch	Aperture / Callus	Columella	Protoconch & Whorls	Umbilical Zone
1. Cueva de Aso, Huesca, Spain (RMNH. MOL.234137) paratype (pt)	Occasional irregular low blunt ribs from 3rd whorl; tendency towards costate.	Ovate, obliquely aligned; peristome slightly/evenly thickened; parietal callus short and thick.	Cylindrical, centrally aligned; moderately thick; torsion equally distributed; intermittent bulges.	Protoconch bulbous; tight, narrow coiling of low, convex whorls; aperture rim not reflected.	End of whorl very short and compact; straight alignment; very narrow umbilical depression.
2. Turziloa cave (NMBE-0590056-1) (AJC-2493-1)	Same as paratype	Ovate, vertically aligned; peristome slightly thicker than pt.; callus same as pt..	Cylindrical, centrally aligned; torsion same as pt.; slight lamellar bulge under penultimate whorl.	Protoconch damaged; widened coiling of last 1/4 of final whorl; aperture with small indentation and reflected from spire.	End of whorl not compact; extending to right side of large umbilical depression.
3. Turziloa cave (NMBE-0590056-2) (AJC-2493-2)	Same as paratype	Ovate, obliquely aligned; peristome slightly damaged; thickness same as pt.; parietal callus smaller than pt..	Cylindrical, centrally aligned; torsion same as pt..	Protoconch bulbous; tight, narrow coiling; compact coiling of last 1/4 of final whorl; rim not reflected.	End of whorl very short and compact; straight alignment; umbilical depression larger than pt..
4. Burgosa cave (NMBE-0590057-1) (AJC-2495-1)	Occasional irregular low blunt ribs from 3rd whorl; subtle horizontal banding.	Rounder than ovate; vertically aligned; thickness and callus as in pt.	Cylindrical, centrally aligned; slightly thinner; torsion same as pt..	Protoconch bulbous; tight, narrow coiling; compact coiling of last 1/4 of final whorl; rim not reflected.	End of whorl short and compact as in pt.; straight alignment; small umbilical depression as in pt..
5. Burgosa cave (NMBE-0590057-2) (AJC-2495-2)	Occasional irregular low blunt ribs; broad horizontal banding on penultimate and body whorl.	Ovate; vertically aligned; callus deformed, less thick/regular than pt.; parietal-palatal indentation.	Cylindrical, centrally aligned; slightly thinner; torsion same as pt..	Protoconch bulbous; tight, narrow coiling; compact coiling of last 1/2 of final whorl; aperture rim reflected.	End of whorl not compact; extending to right side of umbilical depression.
6. Urio gaina cave (NMBE-0590058-2) (AJC-2496-1)	Irregular low blunt ribs from 3rd whorl; apparent horizontal banding on body whorl.	Ovate, obliquely aligned; peristome thick as pt.; callus longer and less thick as pt..	Cylindrical, centrally aligned; thinner; slight lamellar formation under penultimate whorl.	Protoconch bulbous; tight, narrow coiling; equally compact coiling of final whorl; aperture rim not reflected.	End of whorl short and compact as in pt.; straight alignment; elongated umbilical depression.
7. Urio gaina cave (NMBE-0590058-2) (AJC-2496-2)	Shell irregularly smooth; irregular horizontal banding on penultimate and body whorl.	Roundish-ovate; vertically aligned; callus thicker and higher on body whorl than pt..	Cylindrical, centrally aligned; thinner and irregularly smooth.	Protoconch bulbous; tight, narrow coiling; equally compact coiling of final whorl; aperture rim not reflected.	End of whorl short and compact as in pt.; slightly curved alignment; elongated umbilical depression.
8. Cave ES102 (NMBE-0590059-1) (AJC-2498-1)	Occasional irregular low blunt ribs; irregular horizontal banding on body whorl.	Roundish-ovate; vertically aligned; callus thicker/ higher on body whorl than pt..	Cylindrical, centrally aligned; torsion same as pt..	Protoconch bulbous; tight, narrow coiling; equally compact coiling of final whorl; aperture rim slightly reflected.	End of whorl not compact; extending to right side of shell, forming large umbilical depression.

Locality & Specimen	Teleoconch	Aperture / Callus	Columella	Protoconch & Whorls	Umbilical Zone
9. Cave ES102 (NMBE-0590059-2) (AJC-2498-2)	Occasional irregular low blunt ribs; pronounced irregular broad horizontal banding.	Round; vertically aligned; peristome thick as pt.; parietal callus minimal.	Same as pt.; slight lamellar formation under penultimate whorl.	Protoconch bulbous; tight, narrow coiling; slightly compact final whorl; aperture rim slightly reflected.	End of whorl very short and compact; straight alignment; very narrow umbilical depression.

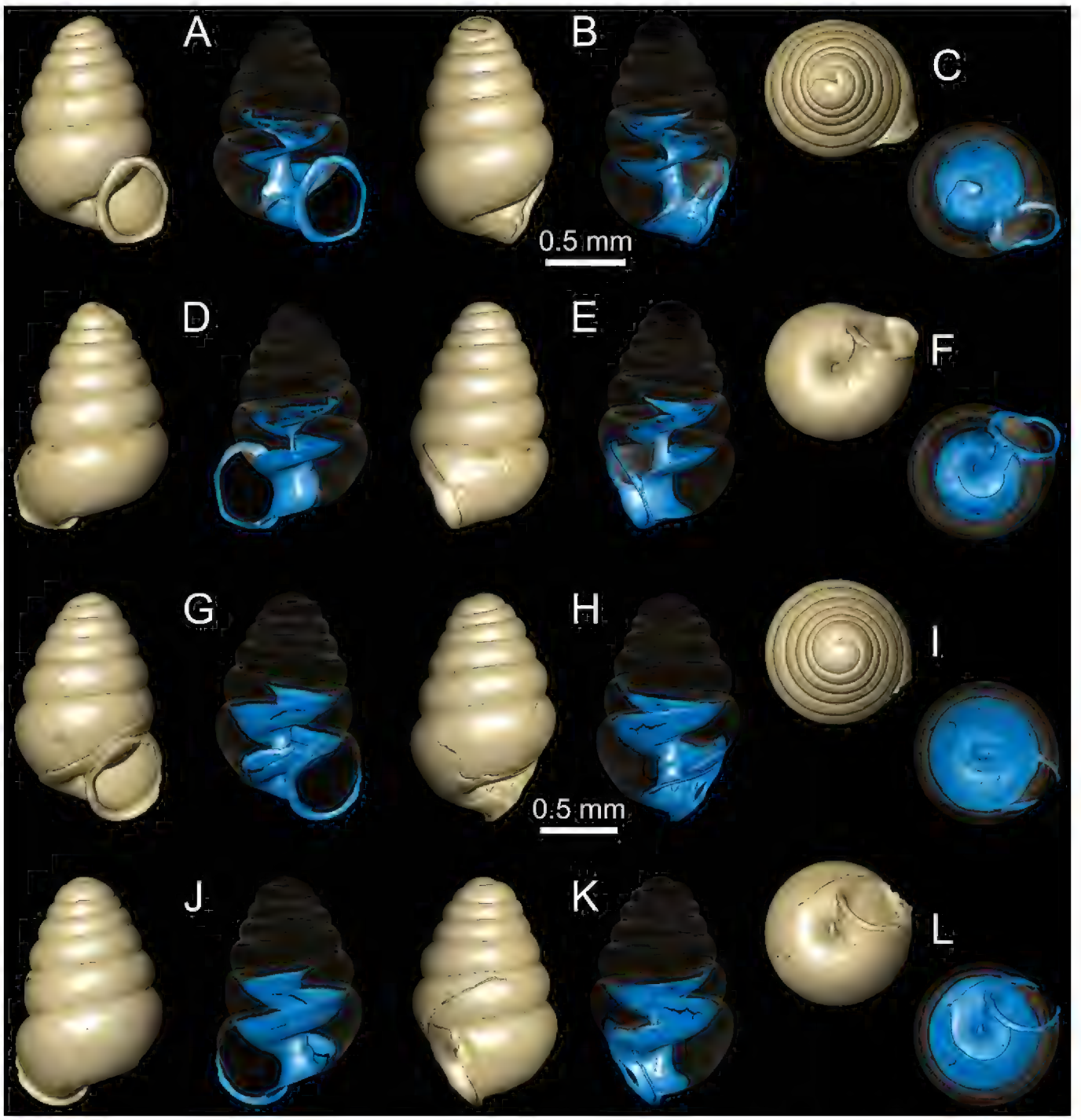

Figure 6. 3D visualizations of Micro-CT data of *Iberozospeum bellesi* shells from Turziloa cave. Specimen NMBE-MOLL-0590056-1 (AJC-2493-1) **A–F.** In opaque and semi-translucent reconstructions in frontal view showing aperture alignment (**A**), aperture facing right view showing columellar configuration (**B**), apical view showing protoconch and coiling tightness (**C**), dorsal view showing columellar structure (**D**), aperture facing left view showing aperture rim alignment (**E**), umbilical view showing umbilical depression and configuration of last ¼ whorl growth and alignment (**F**); **G–L.** 3D visualizations of Micro-CT data of shell specimen NMBE-MOLL-0590056-2 (AJC-2493-2) showing the studied perspectives. Frontal view (**G**), aperture facing right view (**H**), apical view (**I**), dorsal view (**J**), aperture facing left view (**K**), umbilical view (**L**).

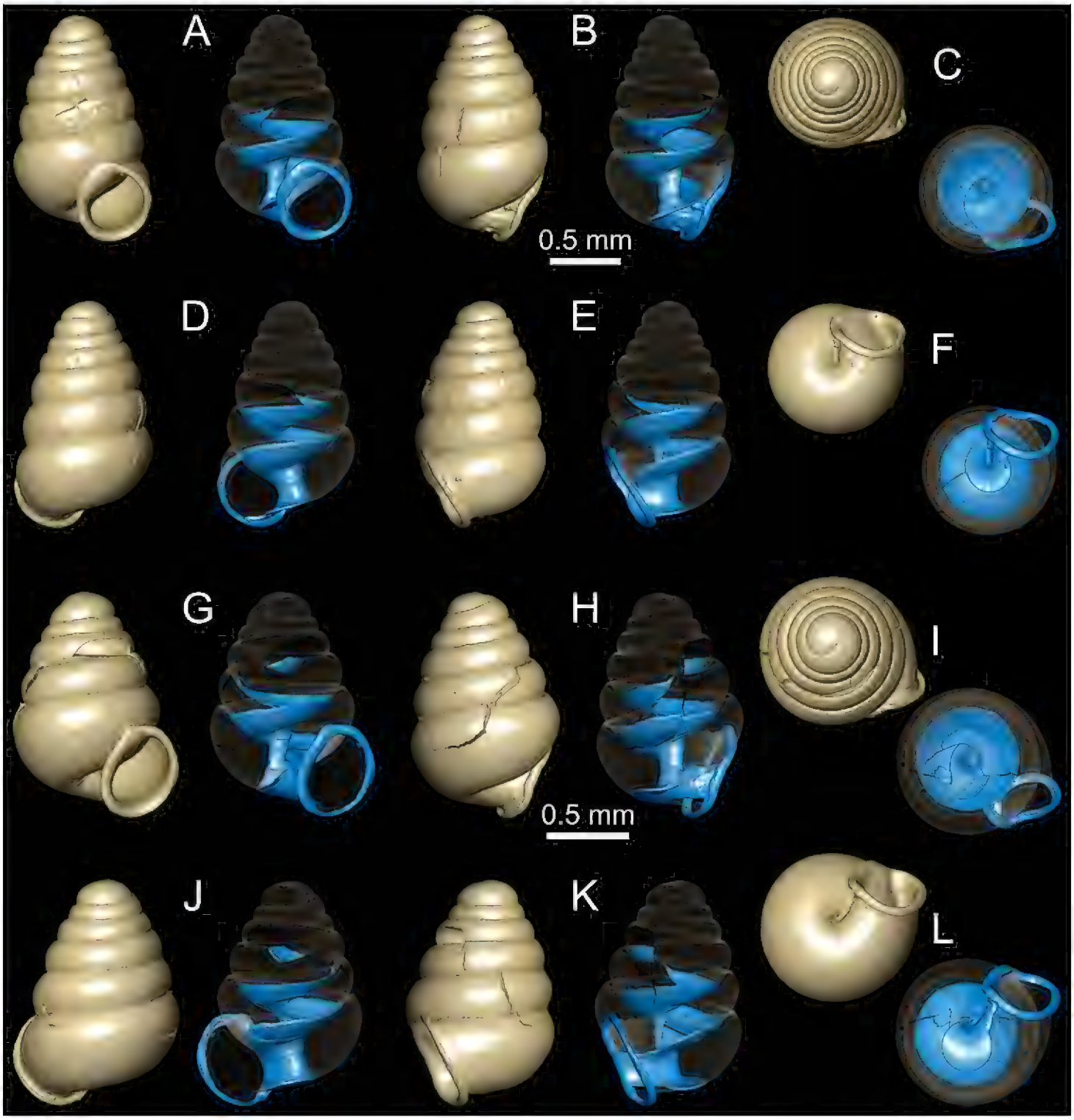

Figure 7. 3D visualizations of Micro-CT data of *Iberozospeum bellesi* shells from Burgosa cave. Specimen NMBE-MOLL-0590057-1 (AJC-2495-1) **A–F.** In opaque and semi-translucent reconstructions in frontal view showing aperture alignment (**A**), aperture facing right view showing columellar configuration (**B**), apical view showing protoconch and coiling tightness (**C**), dorsal view showing columellar structure (**D**), aperture facing left view showing aperture rim alignment (**E**), umbilical view showing umbilical depression and configuration of last ¼ whorl growth and alignment (**F**); **G–L.** 3D visualizations of Micro-CT data of shell specimen NMBE-MOLL-0590057-2 (AJC-2495-2) showing the studied perspectives. Frontal view (**G**), aperture facing right view (**H**), apical view (**I**), dorsal view (**J**), aperture facing left view (**K**), umbilical view (**L**).

(NMBE-MOLL-0585639) provided a complete DNA sequence data set, resulting in a new alignment of the lineages defining the *I. bellesi* clade ("blue clade") in Kneubühler et al. 2022.

To our knowledge, the MHNG houses the only known *I. bellesi* shells from multiple caves in the Pyrénées-Atlantiques, collected by the Swiss speleographer Reno

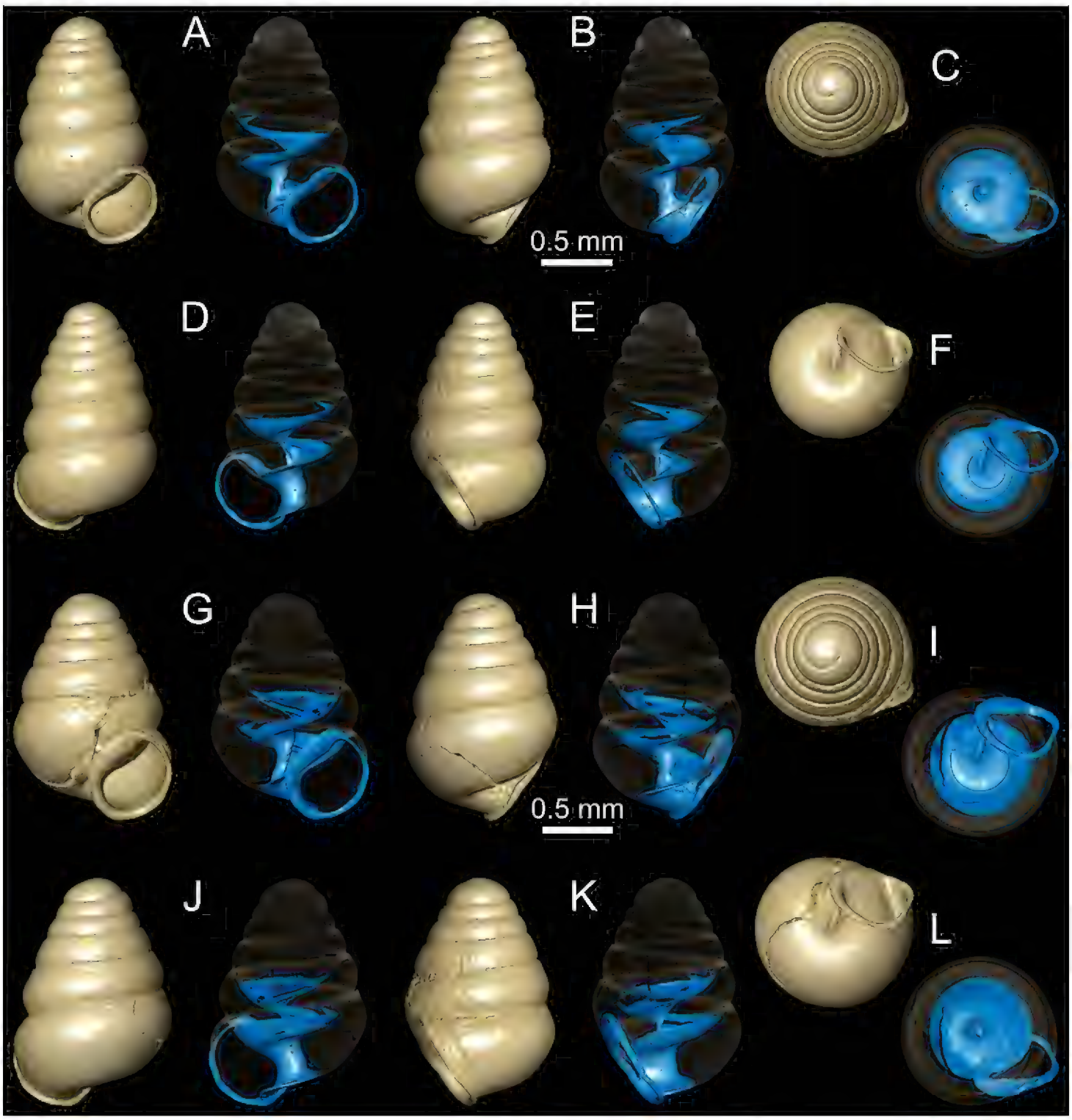

Figure 8. 3D visualizations of Micro-CT data of *Iberozospeum bellesi* shells from Urio gaina cave. Specimen NMBE-MOLL-0590058-1 (AJC-2496-1) **A–F.** In opaque and semi-translucent reconstructions in frontal view showing aperture alignment (**A**), aperture facing right view showing columellar configuration (**B**), apical view showing protoconch and coiling tightness (**C**), dorsal view showing columellar structure (**D**), aperture facing left view showing aperture rim alignment (**E**), umbilical view showing umbilical depression and configuration of last ¼ whorl growth and alignment (**F**); **G–L.** 3D visualizations of Micro-CT data of shell specimen NMBE-MOLL-0590058-2 (AJC-2496-2) showing the studied perspectives. Frontal view (**G**), aperture facing right view (**H**), apical view (**I**), dorsal view (**J**), aperture facing left view (**K**), umbilical view (**L**).

Bernasconi between 1979 and 1985. Dupré (1991) collected shells from 9 French cavities and later (Dupré 1994) cited his collecting "transparent" shells ("ident. BERNUSCONI") from cavities in France, referring to the same material he and Bernasconi collected in 1985. The latter report suggests the shells represented recent mortalities at

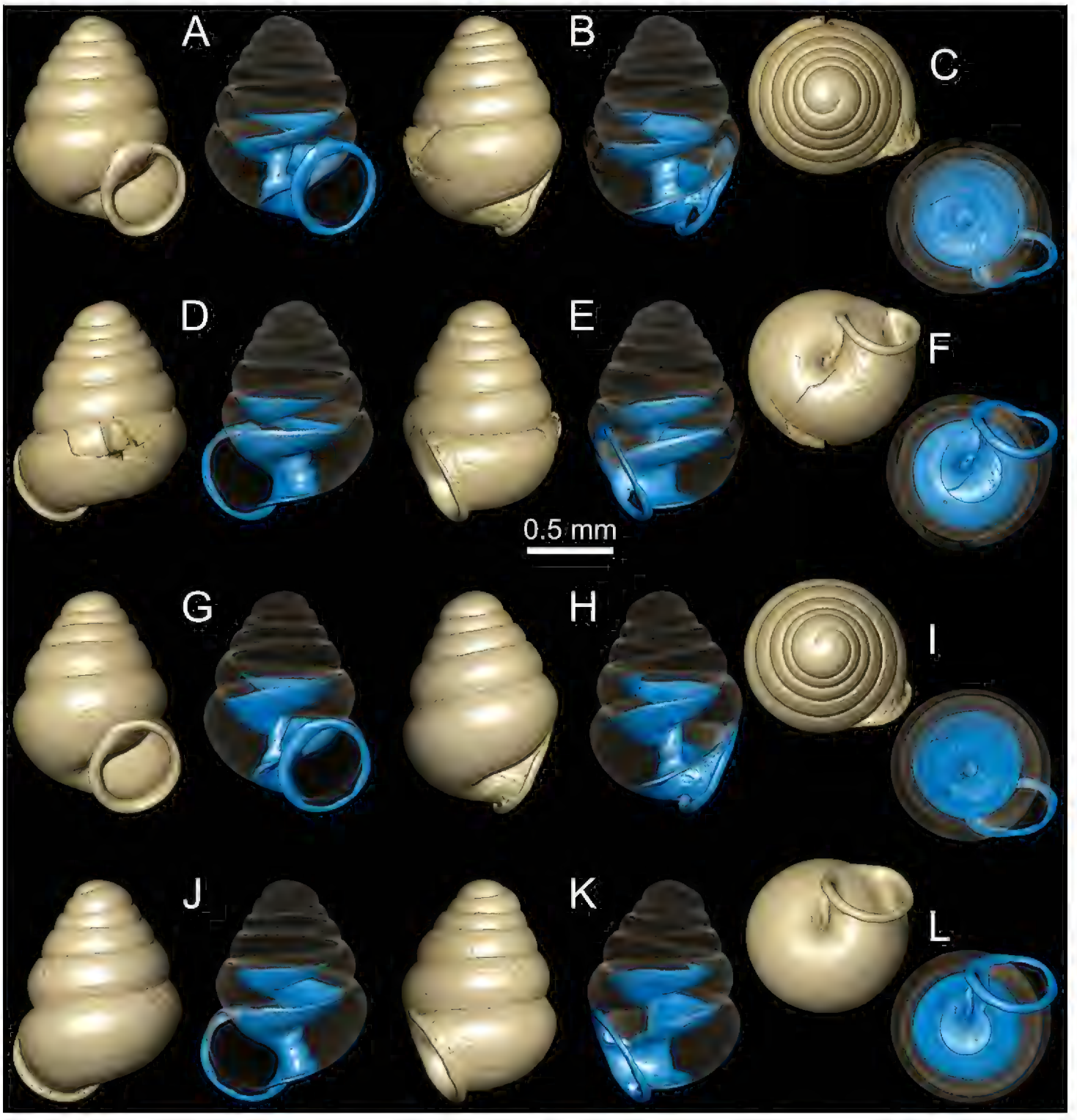

Figure 9. 3D visualizations of Micro-CT data of *Iberozospeum bellesi* shells from cave ES102. Specimen NMBE-MOLL-0590059-1 (AJC-2498-1) **A–F.** In opaque and semi-translucent reconstructions in frontal view showing aperture alignment (**A**), aperture facing right view showing columellar configuration (**B**), apical view showing protoconch and coiling tightness (**C**), dorsal view showing columellar structure (**D**), aperture facing left view showing aperture rim alignment (**E**), umbilical view showing umbilical depression and configuration of last ¼ whorl growth and alignment (**F**); **G–L.** 3D visualizations of Micro-CT data of shell specimen NMBE-MOLL-0590059-2 (AJC-2498-2) showing the studied perspectives. Frontal view (**G**), aperture facing right view (**H**), apical view (**I**), dorsal view showing broad banding on penultimate and body whorl (**J**), aperture facing left view (**K**), umbilical view (**L**).

the time and thus, we infer that the respective caves harboured thriving *I. bellesi* populations. The shells at the NHMG are likely those reported by Dupré (1991).

Known diagnostic characters of *Z. bellesi* include the conical spire, oval aperture with thick parietal callus, spiral sculpture and a columella lacking lamellae

Figure 10. Cave ES102. Entrance of cave (**A**). QW and SG collecting *Iberozospeum bellesi* in situ (**B**).

(Gittenberger 1973; Prieto and Zuazu 2018). In their study of *I. bellesi*, Prieto and Zuazu (2018) remarked no significant differences in the holotype nor in shells they documented from the French Basque Country. The taxonomic examinations in this study corroborate these authors' observations while underscoring the significance of the columella as a key diagnostic character in iberozospeid species. Its continuity in shape (cylindrical), moderate thickness (in aperture facing right view), and characteristic tortional form (irregular bulging under penultimate whorl and above the umbilical depression) is reliably conform in all four populations of *I. bellesi* in this study as well as in the paratype material (RMNH.MOL.234137) from Cueva [del Molino] de Aso, Huesca, Spain.

Autecologically, while empty *I. bellesi* shells are frequently found in large numbers in various parts of cavities and often result from displacement due to flooding events (Lukeneder et al. 2024), living populations remain relatively localized. These findings not only corroborate those reported by Jochum et al. (2012) in caves of the Spanish Basque Country and in Burgos province, but they also show that neither the size, the altitude nor the structure of the karst network appear to be determining factors for the presence of living *Iberozospeum* in the western Pyrenees. Studies are in progress to better characterize the ecological parameters (humidity, temperature, air circulation) that determine the distribution and habitat requirements of this troglobiont species.

Conclusion

This work confirms that *I. bellesi* demonstrates a genetically reliable but ecophenotypically variable species in multiple caves in the Pyrénées-Atlantiques of France.

Our analyses corroborate earlier investigations of *I. bellesi* in the Basque Country (Prieto and Zuazu 2018). Our additional investigation of shell measurements reveals a broad range of spire angles, influencing shell sizes and shape across populations from different caves.

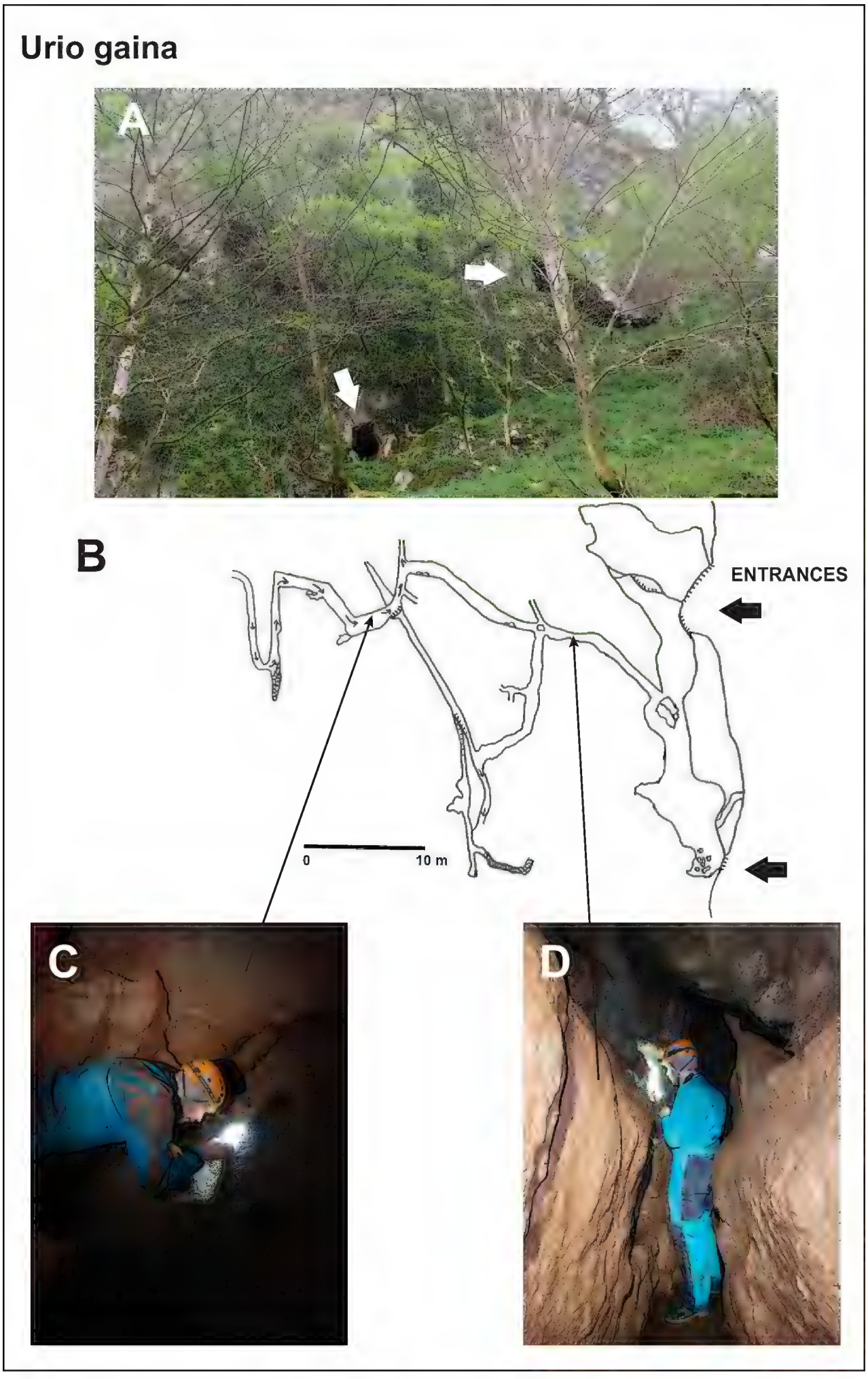

Figure 11. Urio gaina cave. Multiple cave entrances indicated by white arrows (**A**). Diagram of cave passages and chambers with black arrows pointing to the entrances (**B**). QW extracting shells from moist, muddy cave walls (**C, D**).

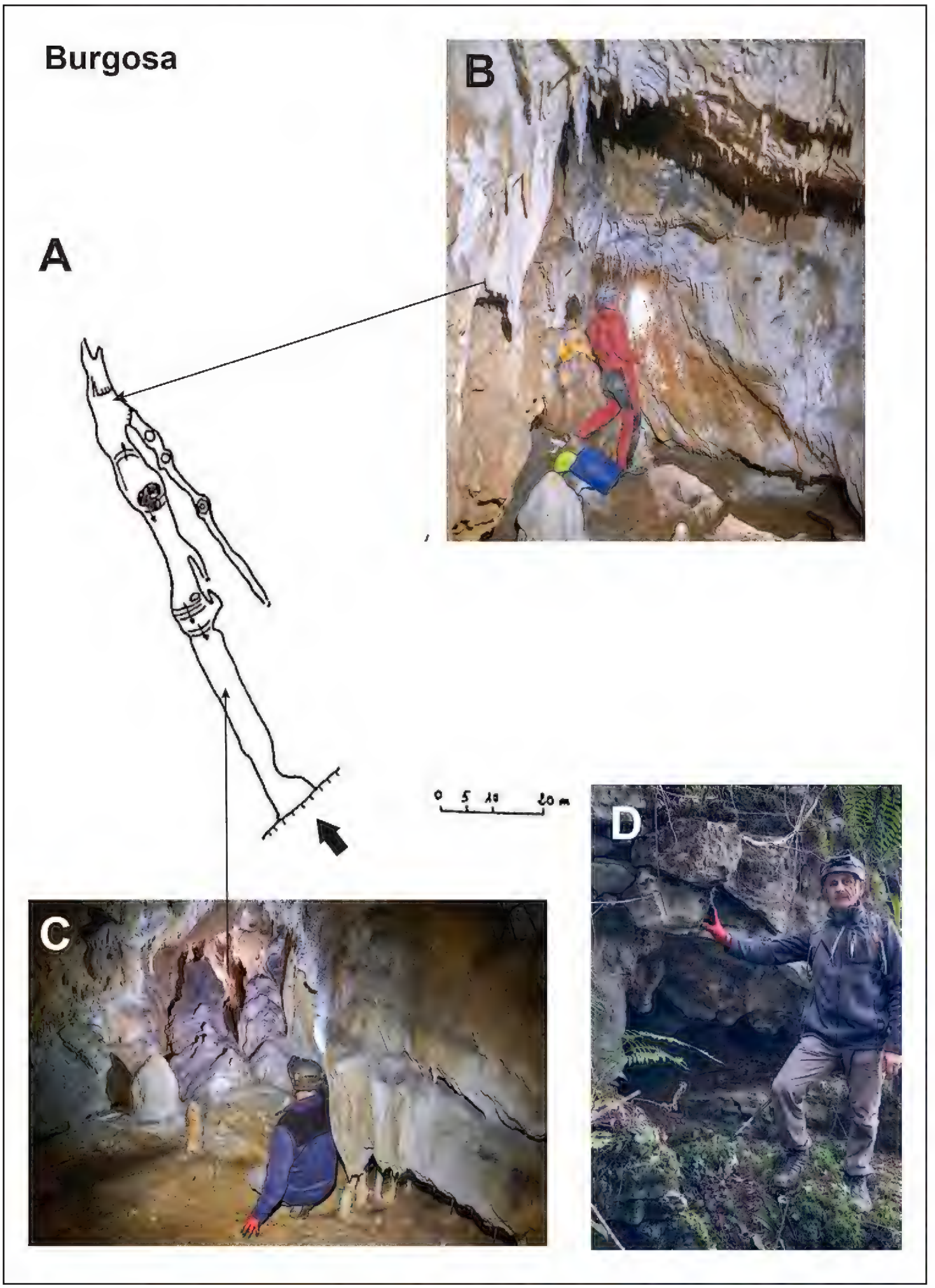

Figure 12. Burgosa cave. Diagram of cave passages and chambers with long arrows showing the collection sites and the short arrow indicating the entrance to the cave (**A**). JC inspecting walls for shells at the back of cave chamber (**B, C**). Entrance with speleologist, Serges Planes in front (**D**).

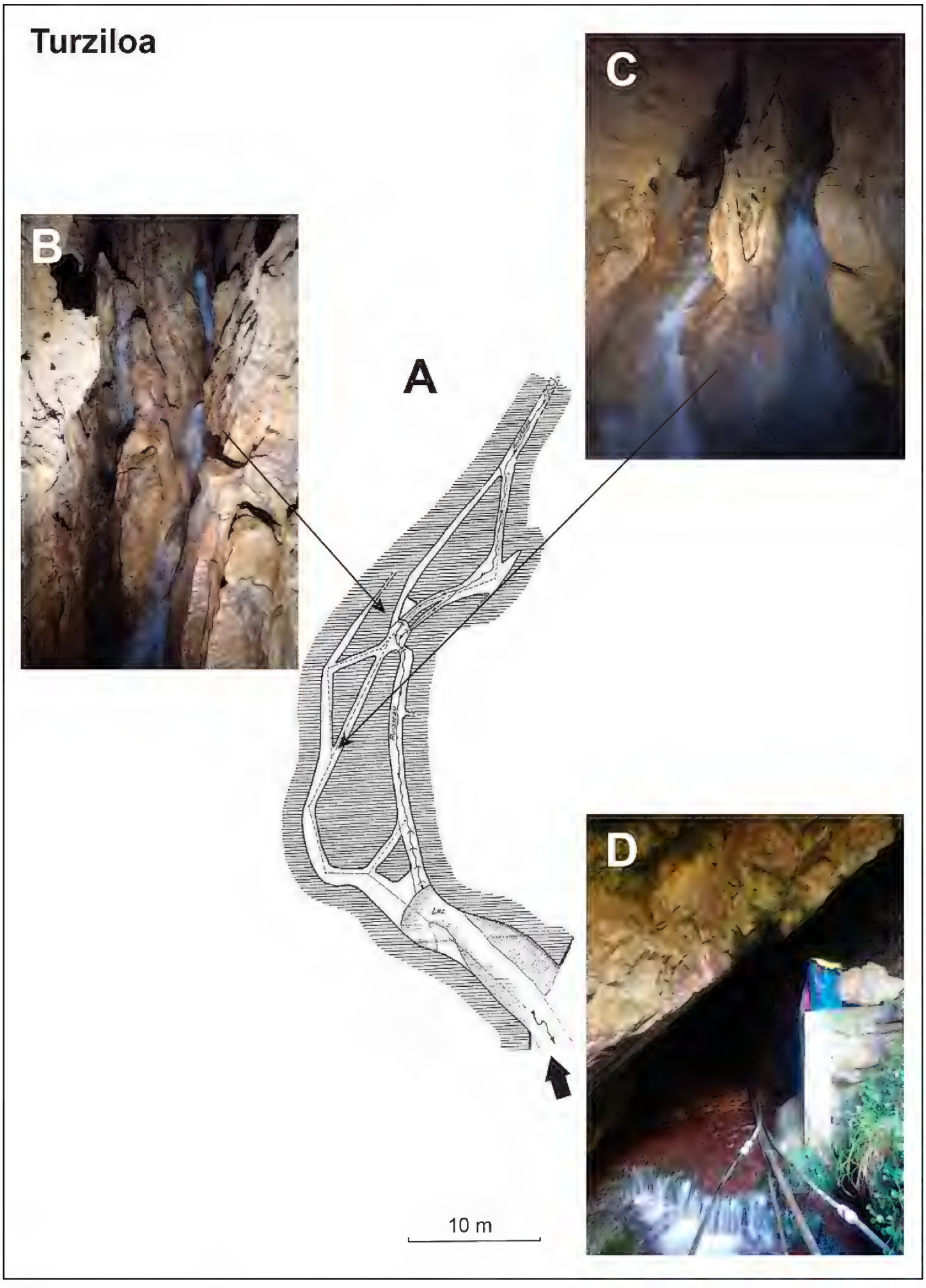

Figure 13. Turziloa cave. Diagram showing cave passages and water flows designated by long arrows and with short black arrow designating the entrance to the cave (**A**). Water cascading down cave walls (**B, C**). Entrance to cave with water collecting pipes used for agricultural purposes (**D**).

For example, cave ES102 harbours smaller shells, high spire angles and stouter morphs. Urio gaina harbours taller shells with lower spire angles and more elongated morphs. Turziloa and Burgosa caves appear to harbour individuals with intermediate shell forms. These differences likely reflect influences of environmental factors (i.e. slender shells living in narrow crevices and squat shells for stability on steep, slippery cave walls), instances of genetic divergence (Schilthuizen et al. 2006), or locally adaptive processes (Proćków et al. 2022).

Our investigations underscore the reliability and continuity of the columella as a species-specific diagnostic character while whorl number and size measurements remain too variable and reflect rather, factors conducive to the development of ecophenotypes. The multifarious topography and hydrogeologically diverse subterranean systems of the Pyrénées-Atlantiques harbour ample microhabitats, driving ecophenotypic variation in *I. bellesi*.

Author contributions

AJ, SG, QW, JC conceptualized the research. The authors, SG, JC, and QW collected and documented the material. JK and EN performed the molecular analyses. PM and BR conducted the 3-D X-ray tomographic imaging and rendering. AJ wrote the first draft of the manuscript. AJ, JC, QW, JK, EN and BR wrote parts of the manuscript; SG, JC and QW funding acquisition; EN provided infrastructure for the research; all authors reviewed and approved the final version of the manuscript.

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Conflict of interest

The authors have no conflicting interests.

Artificial Intelligence (AI) Use

AI assistance was used to format Table 4.

Data availability

All data is published in the manuscript. Sequences are deposited in GenBank.

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